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13. SUPPLEMENTARY NOTES

14. ABSTRACT: Prostate specific antigen screening for prostate cancer is inexpensive, non-invasive and sensitive, but lacks specificity. The accuracy of confirmatory prostate biopsy is only 52% due to either the absence of tumor or the inability to precisely sample small tumors with the biopsy needles. Thus, there is an urgent need to develop methods to accurately image cancers within the prostate, to rule out cancer in men with false positive PSA elevation and to ensure successful biopsy for those with small cancers. Photoacoustic imaging is an emerging functional imaging technique that can detect and diagnose prostate cancer based on the near-infrared optical absorption of either endogenous tissue constituents or exogenous contrast agents. Although endogenous tissue constituents show promise, in order to implement photoacoustic imaging in the clinic, there is a need for increased tumor cell specificity, sensitivity and depth of imaging. To enhance the application of photoacoustic imaging for the detection of early stage prostate cancer, development of near infrared dyes - labeled RNA aptamer that recognizes the prostate specific cell surface protein - prostate specific membrane antigen is proposed to specifically image prostate-cancer. The design incorporates a high energy tunable laser as the source and an ultrasound linear array to detect the acoustic-lens-focused photoacoustic signals generated from the cancerous lesions within the prostate.

15. SUBJECT TERMS
Photoacoustic Imaging, Prostate cancer, Near-infrared, targeted molecular imaging agents

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Table of Contents

1. INTRODUCTION:

Prostate specific antigen (PSA) screening for prostate cancer (PrCa) is inexpensive, non-invasive and sensitive, but lacks specificity. The accuracy of confirmatory prostate biopsy is only 52% due to either the absence of tumor or the inability to precisely sample small tumors with the biopsy needles. Thus, there is an urgent need to develop methods to accurately image cancers within the prostate, to rule out cancer in men with false-positive PSA elevation and to ensure successful biopsy for those with small cancers. Photoacoustic imaging (PAI) is an emerging functional imaging technique that can detect and diagnose prostate cancer based on the near-infrared (NIR) optical absorption of either endogenous tissue constituents (such as deoxyhemoglobin (dHb), oxyhemoglobin (HbO_2), lipid and water) or exogenous contrast agents. Although endogenous tissue constituents show promise, in order to implement PAI in the clinic, there is a need for increased tumor cell specificity, sensitivity and depth of imaging. To enhance the application of PAI for the detection of early stage prostate cancer, development of a NIR dye - labeled RNA aptamer that recognizes the prostate specific cell surface protein - prostate specific membrane antigen (PSMA) is proposed to specifically image PrCa. Two significant innovations are proposed to enhance the application of PAI for the detection of early stage PrCa: 1. Use of a NIR dye labeled RNA aptamer that recognizes the prostate specific cell surface protein PSMA to specifically image PrCa and 2. Optimization of multispectral PAI to differentiate small volume, early stage PrCa tumors from surrounding normal prostate tissue.

2. KEYWORDS:

Prostate Cancer
Photoacoustic Imaging
Prostate specific membrane antigen
Targeted Molecular Imaging Agent
Endogenous
Deoxyhemoglobin
Oxyhemoglobin
Near-infrared
C-scan imaging
Acoustic lens
Laser
Ultrasound
Early stage tumors
Exogenous
Contrast agents
Cancer cell lines
Cancer detection

3. OVERALL PROJECT SUMMARY:

Limitations of endogenous contrast agents for prostate cancer diagnosis. While pre-clinical testing of prostate resection slices indicates that Photoacoustic Imaging (PAI) has higher sensitivity and specificity than TRUS, imaging depth is limited, and chromophores such as de-oxyhemoglobin (dHb) and oxyhemoglobin (HbO_2) have two limitations: i) their small absorptivity factor (extinction coefficient) leads to weak PA signals, limiting the depth of tumor detection as well as the minimal detectable tumor size; and ii) endogenous molecules have little specificity for cancer. In order to improve depth penetration and image quality, exogenous chromophores can be employed to enhance sensitivity relative to endogenous agents. This approach has been demonstrated in the use of a near infrared fluorescent (NIRF) dye, IR800CW conjugated to a peptide that targets the neutropilin-1 receptor for the PAI of breast cancer. Excitation of cells can be optimized by use of a laser tuned to the maximum wavelength of NIR dyes with absorptivity factors two to three orders of magnitude greater than those of endogenous agents. Unlike fluorescence imaging with light as the input and backscattered radiation as the output, PA imaging uses light as the input source for excitation but detection and image formation use ultrasound waves generated by the tissue. Since ultrasonic waves scatter much less than light in the tissue, PAI can produce higher resolution imaging deep in tissue, compared to fluorescent detection. For greater tissue depth penetration and sensitivity, PAI utilizes dyes that absorb in the ‘biological NIR window’ between 700-900 nm. The optimal NIR window is designed to circumvent the strong absorbance of Hb, HbO_2 and H_2O as shown in Fig. 1. NIR dyes related to ICG including Cy7, Alexa750 and IR800CW are ideal and offer a dramatic increase in sensitivity.

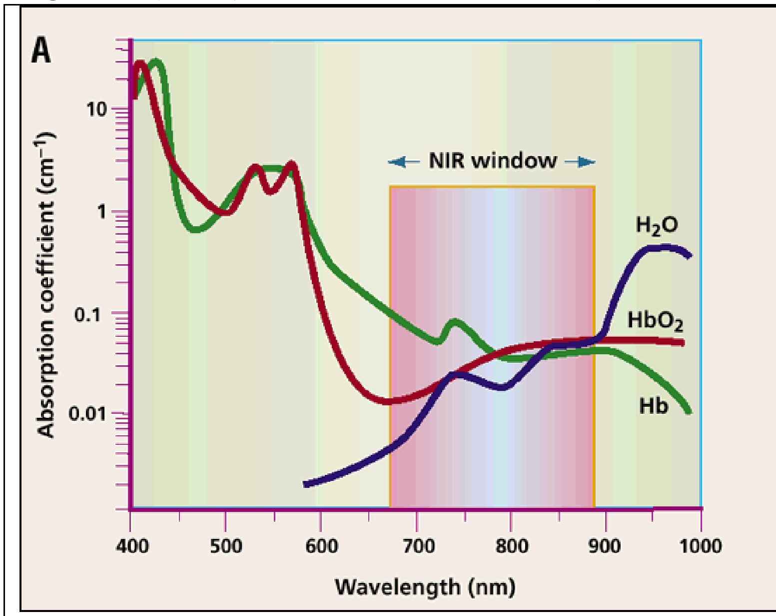

Fig. 1. Biological imaging near-infrared window.

Identification of IRDye800CW as an exogenous contrast agent for PAI. To quantify the PA signal generated with tunable laser excitation in the 700-1000 nm range of endogenous or exogenous dye components, algorithms have been developed to de-convolute the individual chromophore PA images. Five dyes (IRDye800CW, AlexaFluor750, Cy7-NHS-ester, Cy7-sulfo and Dylight800) were tested using our acoustic lens based device at 100 micromolar (μM) concentration. A water bath setup (Fig. 2) was used to determine the photoacoustic spectra of these five dyes. Each contrast agent was interrogated in the NIR region in a five step-interval range and PA signals recorded using our PAI probe. These recorded PA signals were further

processed to find the absorption maxima for each exogenous contrast agent across the NIR region. The PA absorption spectra of each dye have been plotted as shown in the Fig. 3. As

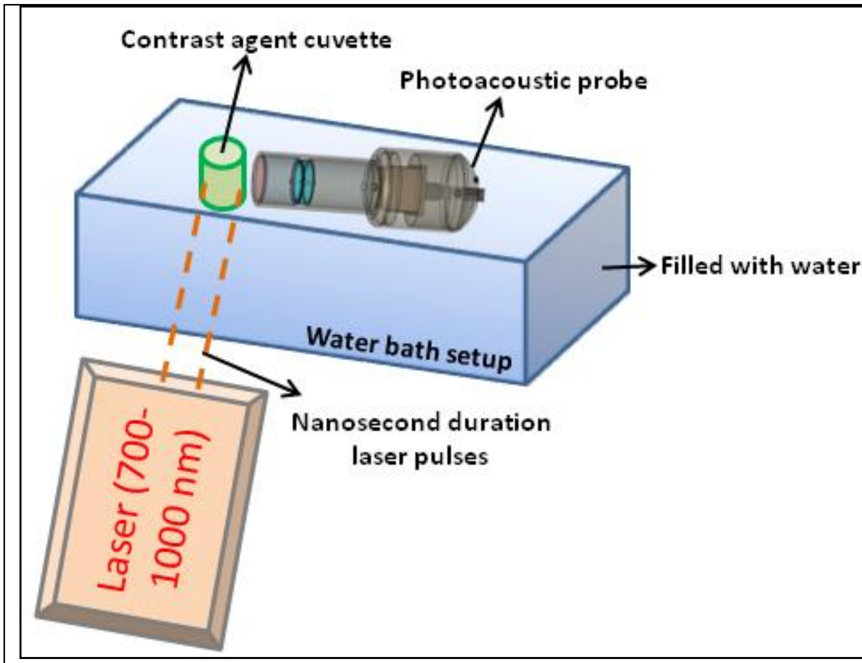

Fig. 2. Spectral analysis of near-infrared dyes. Contrast agents in a cuvette have been examined for a photoacoustic signal using the laser from 700 nm to 1000 nm wavelength.

expected from the reported peak intensities (lambda max) in the ultraviolet-visible spectra, the Alexafluor 750 has a peak PA signal when irradiated at 750 nm, Cy7-NHS-ester at 760 nm, Cy7-sulfo at 755 nm, Dylight800 at 785 nm and the dye IR800CW has a peak signal at 775 nm. When the targeted molecular imaging agents (TMIAs) are injected into the blood stream and PA imaging is conducted during wash out period, the image signal will be the linear sum of signal from all the underlying dominant chromophores (dHb, HbO_2 and NIR dye). It is important to create separate images of individual chromophores to obtain the signature related to only the specific biomarker. This task requires input data from the PA signal spectrum of endogenous or exogenous dye components and algorithms have been developed to de-convolve the individual chromophore images to determine i) their sensitivity by comparing their PA yield (PA signal per micro molar solution), and ii) their spectra shape. Among the five contrast agents/dyes we investigated, IRDye800CW was chosen based on photoacoustic spectrum analysis as determined by the highest intensity achieved relative to the other agents and because the peak absorption is well separated from endogenous tissue constituents, as shown in Figure 3. The initial aim of the project was to use CY7 as the photoacoustic contrast dye. When we started testing, however, we obtained weak photoacoustic intensity with CY7 and needed to search for an alternative dye for a stronger photoacoustic signal [1]. We therefore tested five dyes by both visible absorption spectra and photoacoustic spectroscopy. The un-normalized spectra of the tested five dyes are shown in Fig. 4. The dye showing superior absorption at equimolar concentrations after exposure to room light for three hours was IR800CW followed by Alexa 750 with considerable less absorption by Cy7, sulfo-Cy7 and Dylite. In subsequent results, IR800CW also displayed the best solution stability. Consistent with this data, the IRDye800CW showed better sensitivity and higher photoacoustic signal versus CY7 (Fig. 3). As a result, IRDye800CW was chosen as a replacement for CY7 in subsequent studies as it was shown to provide optimal signal contrast and better stability in handling and the laser compared to the CY7. In addition, IRDye800CW displays distinctive photoacoustic peak absorption at 775 nm which enables better differentiation from endogenous chromophores.

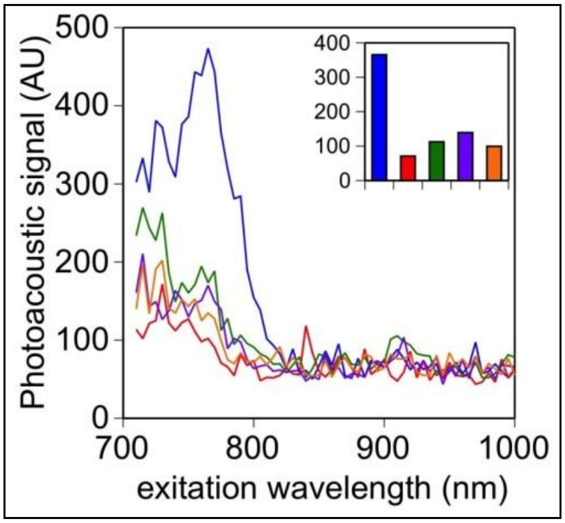

Fig. 3. PA spectra of five contrast agents. IRdye800CW (blue); AlexaFluor750 (red); Cy7-NHS-ester (green); Cy7-sulfo (orange); Dylight800 (purple).

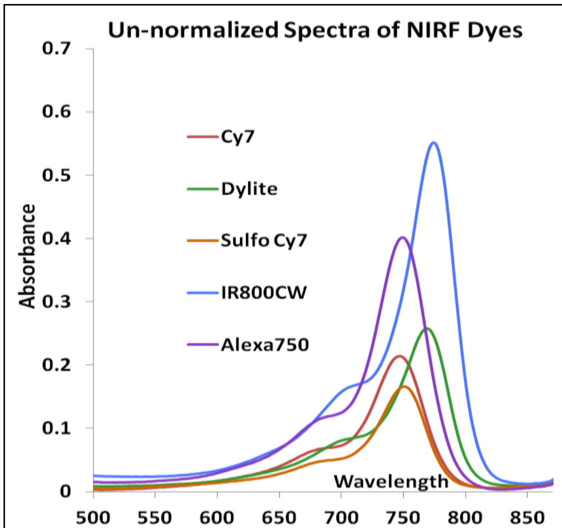

Fig. 4. Optical absorption spectra of five contrast agents. IRdye800CW (blue); AlexaFluor750 (purple); Cy7-NHS-ester (red); Cy7-sulfo (orange); Dylight800 (green).

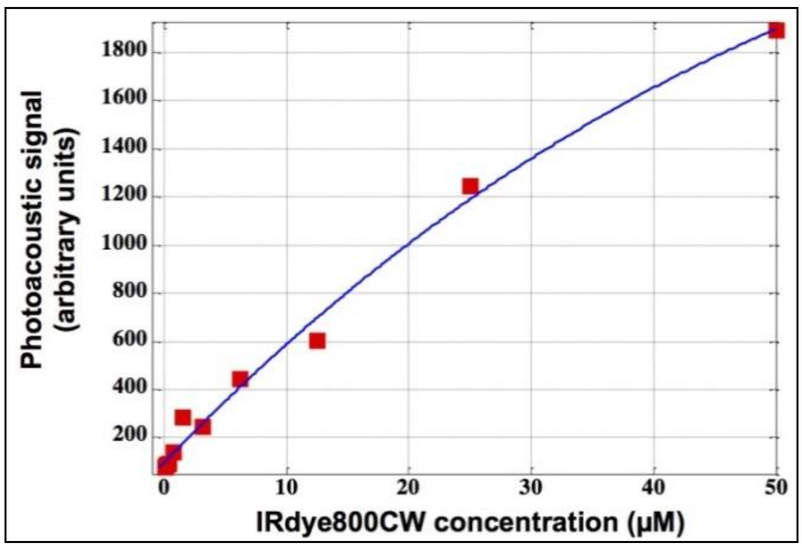

Fig. 5. Sensitivity of IRDye800CW using our PAI device. IRDye800 concentration varied from 90 nM to 50 µM. Each red square represents the corresponding IRDye800CW concentration and its PA signal intensity.

Sensitivity of IRDye800CW using our PAI device. The IRDye800CW was diluted from 50 µM to 90 nM concentrations and photoacoustic signal measured to obtain the sensitivity of the IRDye800CW. Each red square in the plot represents the corresponding IRDye800CW concentration on the x axis and its PA signal intensity on the y axis. PA signal from the dimethyl sulphoxide and de-ionized water has been subtracted from each recorded value and PA signal from the dye alone has been plotted. Using our current PAI system, 90 nanomolar (nM) was determined to be the threshold for signal above noise (Fig. 5).

PAI of PrCa cells using IRDye800 conjugated with A10.3 aptamer bonded to PSMA. As shown in Fig. 3, we identified a NIR dye (IRDye 800CW), which generates a strong PA signal, conjugated it to the PSMA-directed A10-3 aptamer and initiated testing in cell cultures. Prostate

cancer cells lacking (PC3) and presenting (C4-2) the PSMA cell surface protein were used for testing the PA signal from IRDye800CW conjugated to the A10.3 aptamer (dye-aptamer) that binds to PSMA. These two PrCa cell lines were incubated with 4 µM dye-conjugated aptamer, washed thoroughly and centrifuged. The pellets are mixed with agarose, producing a uniform gel phantom for imaging. As shown in Fig. 6, we prepared a total 9 agarose gel phantoms: three with agarose-only; three with (PSMA-) PC3 cells stained with dye-aptamer, and three with (PSMA+) C4-2 cells stained with dye-aptamer, all in a multi-well plate. Initially, the gel phantoms were imaged using a commercially available fluorescence imaging system (IVIS spectrum) at 770 nm to confirm the dye aptamer binding to PSMA (Fig. 6B, D). Once the binding was confirmed, the phantoms were removed from the multi-well plate and placed on the imaging stage as shown in the photograph (Fig. 6A). The PAI scanning procedure (of the yellow square area) was similar to that used in the *ex vivo* imaging of tissue slices, using the prototype instrument. Fig. 6C shows a PA C-scan image of the gel phantoms. The PA signal intensity was increased in the ROI surrounding the C4-2 gel phantoms, demonstrating the presence of IRdye800CW conjugated with A10.3 aptamer bound to cell surface PSMA. Image processing algorithms using MATLAB software were used to reduce the spatial variability and enhance the PA signal intensity throughout the image. Each gel type area was considered as the ROI for obtaining the averaged PA intensity (in future experiments we will determine the signal for individual gels, in order to assess reproducibility). The average PA intensity of the C4-2 gels was ~4 fold higher than either empty phantoms or PC3 cell containing phantoms, and was highly correlated with the fluorescence data obtained by scanning in an IVIS spectrum (Fig. 6D).

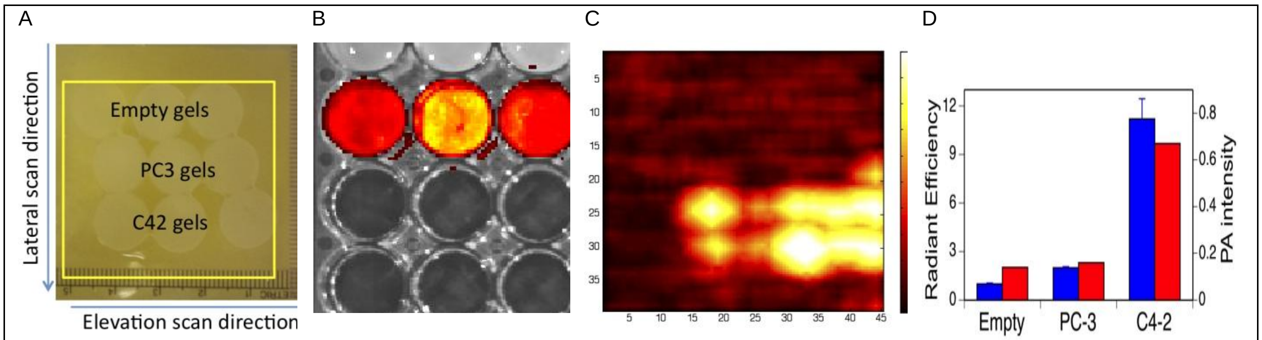

Fig. 6. PAI of PrCa cells embedded inside agarose gels. (A) Digital photograph of imaging platform, with 3 gel types (3 of each, in cylinders). The yellow boxed area was scanned. (B) NIRF (770 nm) IVIS image of the scanned area (C) Photoacoustic image of the scanned area. (D) Average NIRF signal for each cylinder (blue columns) +/-SE, and smoothed averaged PA intensity within an ROI defined by the 3 cylinders of each gel type.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Identification of IRDye800CW as an exogenous contrast agent for PAI.
- Demonstration of increased sensitivity of IRDye800CW versus other labels using our acoustic lens based PAI device.
- PAI discrimination of PrCa cells expressing PSMA (C4-2) versus non-expressing prostate cancer cells (PC3) using IRDye800 conjugated with A10.3 aptamer bonded to PSMA.

5. CONCLUSION:

IRDye800CW was chosen based on photoacoustic spectrum analysis as determined by the highest intensity achieved relative to the other agents and because the peak absorption is well separated from endogenous tissue constituents. PSMA-expressing cells might be used as a biomarker with photoacoustic imaging to classify cancer from normal at an early stage. Our design with the implementation of acoustic lens is so far the best and the most inexpensive way of acquiring time-gated C-scan (coronal) images in real-time.

With successful completion of this research, we will have a device and an accompanying molecular imaging reagent that can be readily moved into human clinical trials. We believe that this work will ultimately yield a real-time, safe and effective imaging device that has a potential to aid in the diagnosis of early stage prostate cancer and reduce patient anxiety (due to the need for biopsy and repeat biopsy) and also reduce the amount of over-treatment of prostate cancer.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

(1) Abstracts:

Quantitative volume determination of normal, neoplastic and hyperplastic mouse prostate using ultrasound imaging (2014) Shalini Singh*, Chunliu Pan*, Ronald Wood[¶,§], Guang-Qian Xiao*, Chiuan-Ren Yeh[§], Shuyuan Yeh[§], Kai Sha*, John J. Krolewski*, Kent L. Nastiuk*. James P Wilmot cancer institute 19th annual scientific symposium. University of Rochester, NY.

Bhargava K Chinni, Shalini Singh, Kent Nastiuk, Hans Schmitthenner, Navalgund Rao, John Krolewski, Vikram Dogra. (2014) **Detecting the sound of light to classify cancer from benign**. James P Wilmot cancer institute 19th annual scientific symposium. University of Rochester, NY.

Lauren Heese, Hans Schmitthenner, Nnamdi Akporji, Michael Regan, Bhargava Chinni, Navalgund Rao, Vikram Dogra. (2015) **Modular Synthesis of Targeted Near Infrared Agents for Photoacoustic Imaging of Cancer**. World Molecular Imaging Congress, September 2-5, Honolulu, Hawaii.

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report.

8. REPORTABLE OUTCOMES:

- Identification of IRDye800CW as an exogenous contrast agent for PAI.
- Demonstration of increased sensitivity of IRDye800CW versus other labels using our acoustic lens based PAI device.

- PAI discrimination of PrCa cells expressing PSMA (C4-2) versus non-expressing prostate cancer cells (PC3) using IRDye800 conjugated with A10.3 aptamer bonded to PSMA.

9. OTHER ACHIEVEMENTS: Nothing to report.

10. REFERENCES:

1. Berlier JE, Rothe A, Buller G, Bradford J, Gray DR, Filanoski BJ, Telford WG, Yue S, Liu J, Cheung CY, Chang W, Hirsch JD, Beechem JM, Haugland RP, Haugland RP. (2003) Quantitative comparison of long-wavelength Alexa Fluor dyes to Cy dyes: fluorescence of the dyes and their bioconjugates. J Histochem Cytochem; 51(12):1699-712. PMID: 14623938

11. APPENDICES:

Quantitative volume determination of normal, neoplastic and hyperplastic mouse prostate using ultrasound imaging: Genetically engineered mouse models are essential to the investigation of the molecular mechanisms underlying human prostate pathology and the effects of therapy on the diseased prostate. Serial *in vivo* volumetric imaging expands the scope and accuracy of experimental investigations of models of normal prostate physiology, benign prostatic hyperplasia and prostate cancer, which are otherwise limited by the anatomy of the mouse prostate. Moreover, accurate imaging of hyperplastic and tumorigenic prostates is now recognized as essential to rigorous pre-clinical trials of new therapies. Bioluminescent imaging has been widely used to determine prostate tumor size, but is semi-quantitative at best. Magnetic resonance imaging can determine prostate volume very accurately, but is expensive and has low throughput. We therefore sought to develop and implement a high throughput, low cost, and accurate serial imaging protocol for the mouse prostate.

Detecting the sound of light to classify cancer from benign: Prostate specific antigen screening for prostate cancer is inexpensive, non-invasive and sensitive, but lacks specificity. The accuracy of confirmatory prostate biopsy is only 52% due to either the absence of tumor or the inability to precisely sample small tumors with the biopsy needles. Thus, there is an urgent need to develop methods to accurately image cancers within the prostate, to rule out cancer in men with false positive PSA elevation and to ensure successful biopsy for those with small cancers. Photoacoustic imaging is an emerging functional imaging technique that can detect and diagnose prostate cancer based on the near-infrared optical absorption of either endogenous tissue constituents or exogenous contrast agents. Although endogenous tissue constituents show promise, in order to implement photoacoustic imaging in the clinic, there is a need for increased tumor cell specificity, sensitivity and depth of imaging. To enhance the application of photoacoustic imaging for the detection of early stage prostate cancer, development of near infrared dyes - labeled RNA aptamer that recognizes the prostate specific cell surface protein - prostate specific membrane antigen is proposed to specifically image

prostate-cancer. The design incorporates a high energy tunable laser as the source and an ultrasound linear array to detect the acoustic-lens-focused photoacoustic signals generated from the cancerous lesions within the prostate.

Modular Synthesis of Targeted Near Infrared Agents for Photoacoustic Imaging of Cancer: Photoacoustic imaging (PAI) is a sensitive, non-invasive means of detecting cancer and is poised to become a transformative method for early screening, targeted biopsies and monitoring disease progression. A key innovation needed in PAI is the design of 'high-gain" imaging agents which can readily be prepared and conjugated to groups which target biomarkers in diseased cells. The current technology relies on using endogenous dyes such as deoxyhemoglobin or oxy-hemoglobin. The goal is to provide a versatile and broadly applicable synthesis of peptide-based agents combining high-gain NIR dyes that may be conjugated to a PSMA inhibitor for the early detection of prostate cancer by PAI. There is a precedent for the use of a targeted exogenous NIR dye, IR800CW, conjugated to a peptide specific for neutropilin yielding a photoacoustic signal.